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14. ABSTRACT
Three established human breast cancer cell lines were analyzed and found to express the L1 adhesion/recognition molecule by immunofluorescence, RT-PCR, and western blotting. Protein expression levels correlated to their known metastatic abilities. Cells released a large soluble proteolytic L1 fragment and tiny L1-containing vesicles into culture media. Breast cancer cells express multiple integrin cell surface receptors that can bind to the released or shed L1. L1 proteolysis can be upregulated by a phorbol ester, indicating that this phenomenon can be modulated. A L1-shRNA retroviral vector was found that attenuated expression of breast cancer cells, and 3 retroviral vectors were constructed that cause expression of different length L1 ectodomain fragments. The chick embryo was found to be a sensitive system for studying breast cancer metastasis by using as few as 5,000 cells injected into the blood stream followed by recovery and culture of cells from the brain.

15. SUBJECT TERMS
Breast cancer, motility, metastasis, adhesion molecule

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Introduction

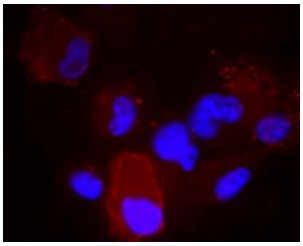
Understanding mechanisms that contribute to breast cancer cell motility should shed light on why this cancer frequently spreads to sites such as brain and why such metastases are so lethal. The purpose of this study was to explore a novel mechanism that may account for the ability of human breast cancer cells to express a form of the L1 adhesion molecule as a means to stimulate their own motility and metastasis. This is still a largely unexplored concept in the progression of breast cancer, and these studies have provided some new insights into the aspects of this disease that contribute to patient mortality. Breast cancer cells metastasize to a variety of secondary sites, including brain, which is ultimately responsible for patient death. A recently discovered mechanism of cleavage of the L1 adhesion molecule results in self-stimulation of motility in ovarian and uterine cancers and causes of high mortality rates. L1 is normally expressed on axons in the brain where it is thought to stimulate growth. This project explored production and cleavage of L1 in established human breast cancer cell lines. L1 was found to be expressed abnormally in breast cancer cell lines. L1 was cleaved and released as a large ectodomain from the cell surface and also in the form of small exosomal membrane vesicles.

Body

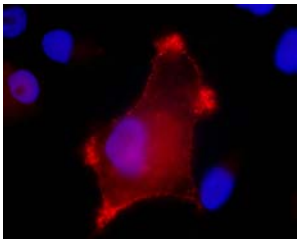
The present work aimed to explore different forms of L1-CAM's potential effects in stimulating cancer cell motility and metastasis. Breast cancer cells can metastasize to a variety of secondary sites, including brain, which usually leads to ultimate patients' death. A recently discovered mechanism of proteolysis or "shedding" of cell adhesion molecule L1 reveals a pattern of autocrine/ paracrine stimulation of cell motility in ovarian and uterine cancers, resulting in high mortality rates. L1 is expressed and shed abnormally in some breast cancer cells, stimulating their motility and metastasis via interaction with focal adhesion molecules, such as integrins.

1. Immunostaining of live and fixed breast cancer cell lines for L1.

MDA-MB-231 and MDA-MB-435 human breast cancer cell lines were immunostained live for cell surface L1 and after fixation and permeabilization for intracellular deposits of L1. Live cells exhibited limited positive staining, indicative of expression of L1 with incomplete proteolysis from the cell surface. Flow cytometry analysis of live cells showed very little staining, correlating with immunocytochemistry.



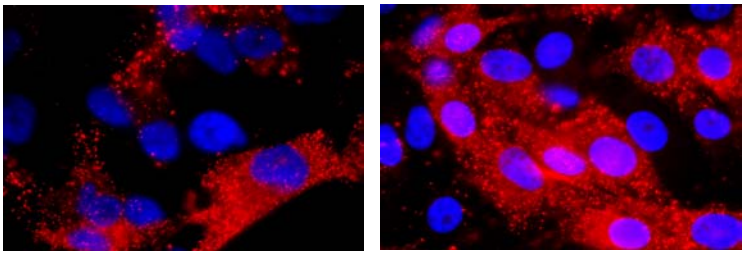
Live MDA-MB-231 cells



Live MDA-MB-435 cells

Figure 1. Live MDA-MB-231 and MDA-MB-435 cells showed only some discrete cell surface staining for L1 (red) upon immunocytochemistry with anti-L1 antibodies. Total nuclei are stained blue. The positive cells shown were atypically bright compared to the vast majority of cells.

Fixed and permeabilized cell lines exhibited numerous bright intracellular puncta after immunostaining for L1. Taken with the results from live cell staining, this showed that these breast cancer cells apparently expressed L1, but most of it was not localized on the cell surface.

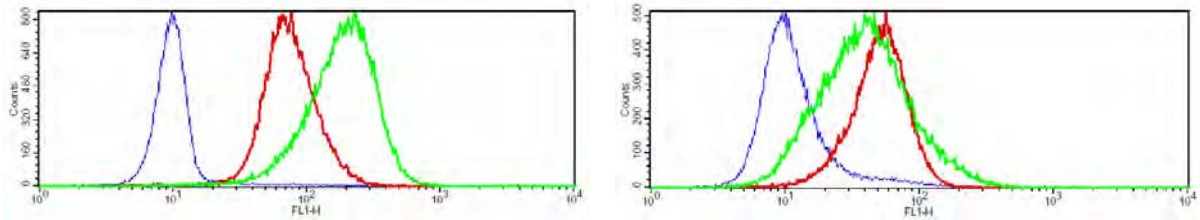


Fixed MDA-MB-231 cells Fixed MDA-MB-435 cells

Figure 2. Fixed and permeabilized MDA-MB-231 and MDA-MB-435 cells showed numerous intracellular punctate staining for L1 (red) upon immunocytochemistry with anti-L1 antibodies. Total nuclei are stained blue.

2. Integrin receptor expression studies.

MDA-MB-231 cells and MDA-MB-435 cells were analyzed by flow cytometry for expression of 2 integrin receptors that are known to bind to L1: αvβ3 and αvβ5. Both cell lines expressed both integrin receptors, but at different levels as shown below.



Integrin Receptor expression in MDA-MB-435 cells Integrin Receptor expression in MDA-MB-231 cells

Figure 3. FACS analysis shows the presence of αvβ5 and αvβ3 integrin receptors on the cell surface of live MDA-MB-435 and MDA-MB-231 cells. The blue line represents the no primary control, the red line represents αvβ5 integrin cell surface expression and the green line represents αvβ3 integrin cell surface expression in 435 and 231 cells respectively.

3. Generation of anti-L1 polyclonal antibodies.

We previously had success using an anti-rat L1 monoclonal antibody to sequester shed L1 ectodomain from the media in cultures of rat glioma cells, and this resulted in reduced motility of the cells. Thus, we wanted to produce a custom polyclonal anti-human L1 antibody that would be effective at blocking the L1 ectodomain with cell surface receptors on human breast cancer cells. This theoretically would work like the Herceptin antibody against breast cancer, only aimed at stopping cell motility. We had New England Peptide company design and produce anti-peptide antibodies against the 6th Ig domain of human L1 in 2 different rabbits. They were affinity-purified against the peptide antigen.

The antibodies were used to immunostain positive control cells transfected with either the mouse L1 or human L1 cDNAs, but FACS analysis and immunocytochemistry did not show positive staining. The antibody was also added to glioma cell cultures in order to block motility, however no decrease in motility was observed. Thus, it appears that this custom polyclonal anti-L1 antibody did not recognize L1 and was not effective at decreasing cancer cell motility.

4. L1 expression is correlated with the motility of breast cancer cells.

A previous paper on breast cancer cell lines' malignant potential (1) revealed that MDA-MB-435 cells metastatic ability is great than MDA-MB-231, which is then greater than MDA-MB-468. Therefore, to test if L1-CAM is correlated with this trend of malignant potential, its expression was examined on RNA and protein levels. Data showed (see figure below) that with increase of malignant potential among the three cell lines, L1 expression is also increased, indicating L1's role in facilitating cell moltility. The expression of L1 in less metastatic cell line MDA-MB-468 and the more metastatic cell lines MDA-MB-435 and MDA-MB-231 was characterized and L1 was found to be overexpressed in highly metastatic cell lines.

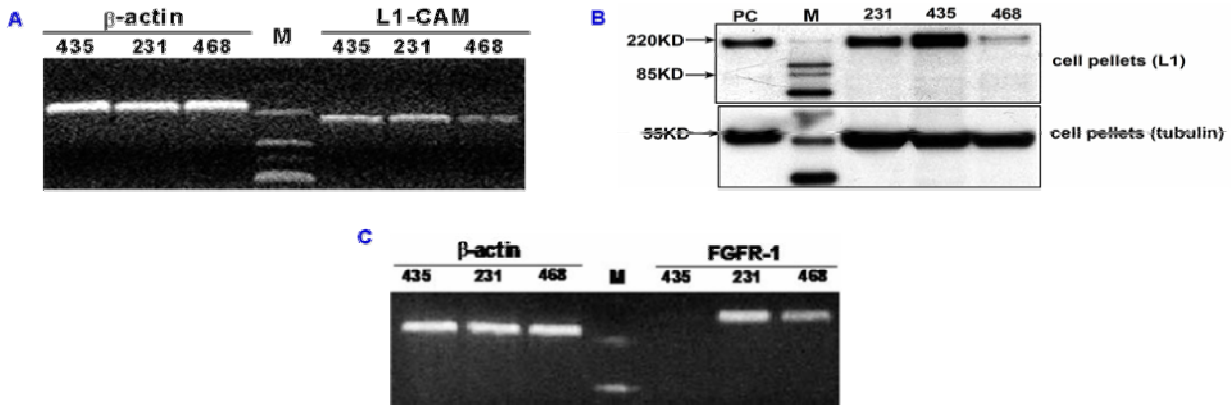


Figure 4 . **L1 expression level is tightly correlated with the metastatic ability of breast cancer cell lines.**

A) RT-PCR result showing L1-CAM's expression in 231- MDA-MB-231; 435- MDA-MB-435 and 468- MDA-MB-468 cell lines, with beta-actin as the internal control.

B) Western blot analysis of full length L1 (220KD) and plasmin cleaved L1 cytoplasmic tail (85KD) expression in cell pellets of the same three breast cancer cell lines, with tubulin as the internal control. The least metastatic cell line MDA-MB-468 showed the least L1 expression. PC- U87 Glioma cells.

C) **FGFR** expression in breast caner cell lines tested. Besides MDA-MB-435 different from the other two as duct carcinoma, **FGFR** expression in MDA-MB-231 and 468 is correlated with L1-CAM.

5. L1 shedding is increased upon PMA activation.

Mechtersheimer etc.(*2*) have shown that L1 shedding by ADAM10 could be induced in L1 over-expressed CHO cells. Whether it is true for endogeneous L1 in breast cancer cells was tested using the same three cell lines in above tests. As presented in Fig.5, PMA (phorbol 12-myristate 13-acetate) did increased L1 shedding in the form of ectodomain then into cell culture media. And according to the L1 molecular weight, this shedding was done by ADAM10.

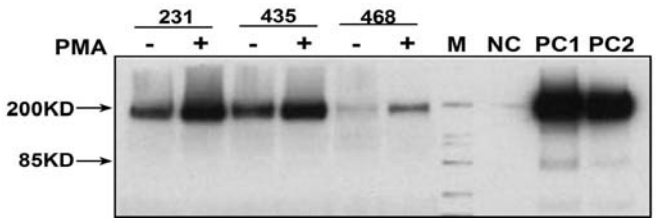


Figure 5. L1 shedding analysis. MDB-MA-231, MDA-MB-435 and MDA-MB-468 cells were stimulated by 100ng/ml PMA in serum free media for 1h, cell culture medium was then precipitated by TCA overnight to get protein pellets for western blot analysis. Shed L1 ectodomain (200KD) were shown in the three cell lines. The least metastatic cell line MDA-MB-468 showed the least L1 ectodomain shedding. NC- QT6 cells; PC- U87 Glioma cells.

The result suggested that further signal transduction pathways were activated by PMA to induce more L1 shedding. This could be through PKC pathways (*3*). Activated PKC would induce more ERK phosphorylation, while MAPK pathway could be the common pathway for N-CAM family downstream signal transduction (*4*). Further investigation on L1's functional pathways is currently under study.

6. L1 is found in secreted exosomal vesicles.

L1 was also released into the culture media in the form of small exosomal vesicles. The biological significance of the secretion of exosomes from any cancer cells is not known and they probably add to the metastatic potential of breast cancer cells. Exosomes were first pelleted by ultracentrifugation of cell culture media and western blotting, followed by visualization using transmission electron microscopy.

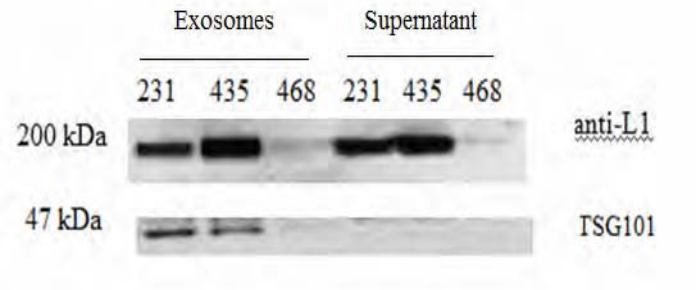


Figure 6: Western blot showing L1 expression in the exosomal pellets and cell supernatant of MDA-MB-231, MDA-MB-435 and MDA-MB-468 cells. Exosomes are isolated from the cells by differential centrifugation. Presence of **exosomes** is confirmed by checking for the exosomal marker protein TSG101. Exosomal L1 is expressed at a low level in MDA-MB-468 (less metastatic) cell line.

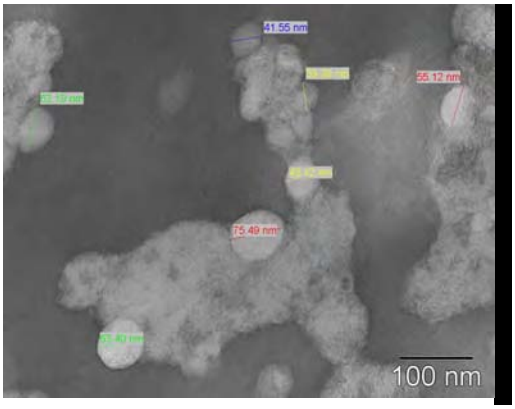


Figure 7: Transmission electron microscopy images of **exosomes** isolated from the media of MDA-MB-435 cells. The isolated **exosomes** can be visualized as small 40-100 nm membrane vesicles. Diameters of a few exosomes are labeled on the figure.

7. Generation of L1-modified breast cancer cell lines.

Lentiviral vectors can be used for 1) overexpressing different fragments of the L1 ectodomain in the less metastatic cell lines and 2) shRNA against L1 for down regulating L1 expression in highly metastatic cell lines. *In vitro* motility analysis of cell behavior can then be done to examine the differences resulted thereafter. *In vivo* studies involve a novel model of chick embryo brain tumor forming assay, which will precisely quantitate the number of breast cancer cells that localize to brain and then to determine metastatic potential between different cell lines. L1 is hypothesized to help facilitate breast cancer cell metastasis into brain.

Correlation of results between the two complementary approaches *in vitro* and *in vivo* will establish if the novel motility assay can predict metastatic or invasive potential of human breast cancer cells. Therapeutic strategies can be devised to inhibit signals transduced from this mechanism which may contribute significantly to breast cancer cell spread and metastasis, including to brain and elsewhere.

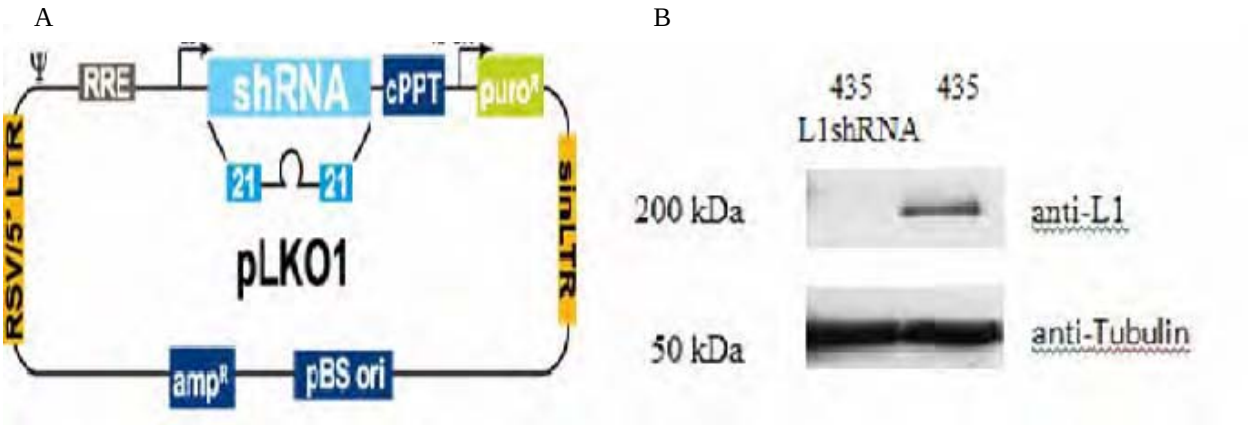


Figure 8. Using shRNA to knock down L1-expression in highly metastatic cells. A) Linear schematic diagram of lentiviral vector pLKO1 from Open Biosystems. B) Western blot showing the L1 expression in MDA-MB-435 cells and MDA-MB-435 infected with L1-shRNA-expressing lentiviral vector, with tubulin as internal control. As shown, L1 expression is greatly attenuated in vector-infected MDA-MB-435 cells.

Since L1 ectodomain was proposed as the key interaction part with other extra-cellular molecules including integrins and FGF or EGF receptors through its RGD or fibronectin III domains (5, 6), this part of L1 was fragmented into three parts for further study. As shown in Fig.6, successful cloning of the three fragments was completed by Hi-fidelity Taq enzyme and then inserted into LVV-1879MCX Lentivirus vector.

Then virus was introduced firstly into 293T or QT6 cells to test correct L1 fragments expression. As shown in Fig. 6 different parts of L1 were successfully expressed in those cell lines at the right size. Currently, we are infecting the least malignant potential breast cancer cell line (MDA-MB-468) with those new vectors, and then will test their ability to facilitate cell motility and metastasis in our *in vitro* and *in vivo* systems. We have infected MDA-MB-231 cells with the L1-shRNA vector, and MDA-MB-468 cells with the full-length L1 ectodomain vector. The new cell lines have been drug selected and are now being tested for altered L1 expression before using them in the assay systems.

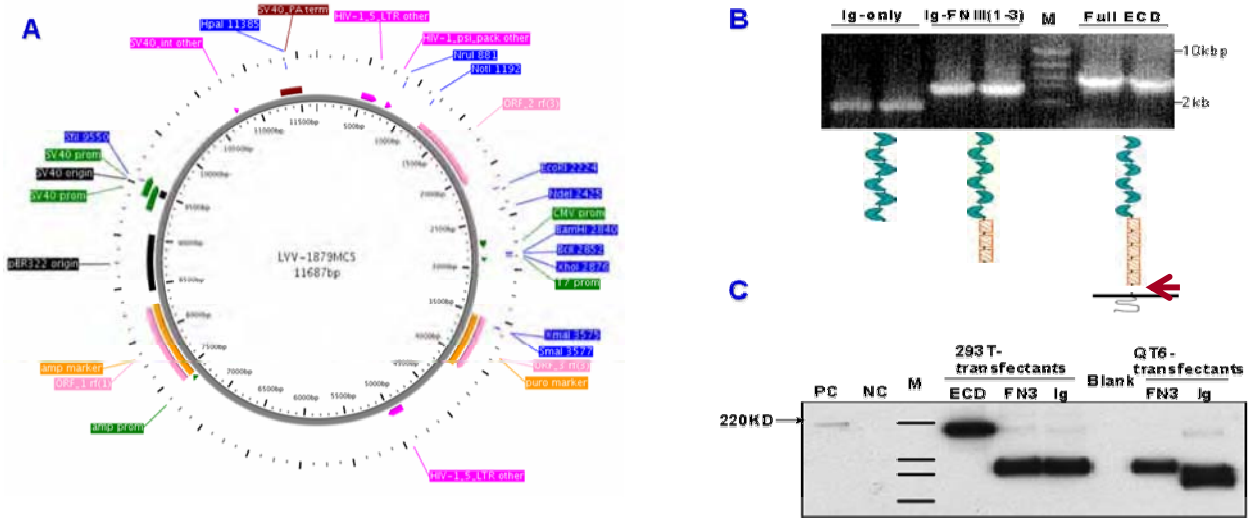


Figure 9. Lentivirus vector construction of different L1 ectodomain fragments and their expression. A) Schematic diagram of the structure of Lentivirus LVV-1879MCX being used. Different L1 ectodomain fragments were cloned respectively into MCS region by SpeI and Xho I restricted enzyme sites. B) PCR cloning of L1 ectodomain fragments. Full ECD is the whole L1 ectodomain without cytoplasmic tail, mimicking L1 after ADAM10 cleavage (red arrow); Ig-FNIII stands is a truncated ectodomain with all Ig domains and FN domains 1-3, and Ig-only is L1 ectodomain without any FNIII repeats. C) Infection and expression of constructed L1-ectodomain lentiviruses in 293T and QT6 cells. Shown as above, the three fragments were expressed successfully in 293T or QT6 cell lines. NC- plain QT6 cells w/o L1 infection; PC- U87 Glioma cells.

8. *In vivo* breast cancer cell injection studies.

We have begun the *in vivo* injection studies. First, 50,000 cells from MDA-MB-231 were injected into the extraembryonic blood vessels of chick embryos on E5. Brains were dissected, dissociated into single cells, and plated in dishes under drug selection at E9. This resulted in approximately 150 cancer cell colonies that arose from cells that had metastasized into the brain. Next, 5,000 cells were injected on E5 to

determine if this low number would still be sufficient to result in metastasis out of the blood stream and into the brain. Our results so far show that brains process similarly to above result in 25-50 cancer cell colonies per brain. This establishes that the chick embryo is a sensitive system to study breast cancer cell metastasis into the brain. This, then, allows us to inject 50,000 cells when we expect a modified cell line (e.g. shRNA-infected 231) to decrease the number of colonies per brain, or to inject 5,000 cells when we expect a modified cell line (e.g. L1 ectodomain vector infected 468 cells) to increase the number of colonies per brain.

Key Research Accomplishments

- Three human breast cancer cell lines express the L1 protein, proteolyze it, and release it as a soluble ectodomain and contained in exosomal vesicles.
- L1 expression level is tightly correlated with the metastatic ability of breast cancer cell lines. The more metastatic cell lines MDA-MB-231 and MDA-MB-435 express much higher levels of L1 protein compared to the less metastatic cell line MDA-MB-468.
- Increased L1 shedding by the ADAM10 protease was induced when all 3 cell lines were incubated in the phorbol ester PMA, which activates protein kinase C. This raises the possibility that this signaling pathway might be a target for therapeutic intervention aimed against L1 shedding.
- Exosomes were successfully visualized using Transmission Electron Microscopy in MDA-MB-435 supernatant after a series of differential centrifugations and were found to be in the expected size range of 40-100nm. These exosomes may serve as another “soluble” form of L1 molecule because of their small size.
- Three lentivirus vectors were constructed for overexpressing the different length L1 ectodomains in less metastatic MDA-MB-468 cells to check if they can increase the motility and metastatic ability of these cells.
- L1 expression was effectively knocked down by specific shRNA expressing lentivirus in the more metastatic MDA-MB-435 cells. These cells will be used to check if this would decrease their motility and metastatic ability.
- Human breast cancer cell lines express multiple L1-binding integrins.
- Injection of 5,000 breast cancer cells into the extraembryonic vasculature still results in metastasis into the brain of the chick embryo. Thus, this *in vivo* model system can be used for our human breast cancer cell lines after modification of L1 expression, either up or down.

Reportable Outcomes

Presentations

Galileo, D.S., K. Chilukuri, Y. Li, and K. Teixeira. Novel quantitation of autocrine stimulation of cell motility *in vitro* and metastasis *in vivo* of human breast cancer cell lines. 5th Department of Defense Era of Hope Meeting, Baltimore, MD, June 2008.

Teixeira, K. and D.S. Galileo. A quantitative analysis of breast cancer metastasis to brain. NIH, NCRR 2nd Biennial National IDeA Symposium of Biomedical Research Excellence (NISBRE), Washington D.C., August 7, 2008.

Teixeira, K. and D.S. Galileo. Quantitative analysis of breast cancer metastasis to brain. University of Delaware 25th Annual Undergraduate Research Symposium, May 3, 2008.

Teixeira, K. and D.S. Galileo. Quantitative analysis of breast cancer metastasis to brain. University of Delaware Summer Undergraduate Research Symposium, August 13, 2008.

Teixeira, K. and D.S. Galileo. Quantitative analysis of breast cancer metastasis to brain. 11th Annual Undergraduate Research Symposium in the Chemical and Biological Sciences. Univ. of Maryland Baltimore County. October, 2008. First Place Winner Biological Sciences 4 poster competition.

Cell Lines

1) MDA-MB-435 breast cancer cells infected with the L1 shRNA lentiviral vector. These cells have attenuated L1 expression compared to the parental cells. These cells are also puromycin resistant.

Other modified breast cancer cell lines (231 and 468) are currently being made and tested.

Conclusions

The characterization of L1 in the different breast cancer cell lines and correlating the levels of L1 with their known metastatic ability suggests a possible mechanism by which L1 could influence the cell motility and metastatic potential of breast cancer cells. Overexpression and attenuation of L1 in the appropriate cell lines using our vectors and performing *in vitro* and *in vivo* analyses will establish how L1 is involved in cell motility of these breast cancer cells. Exosomes released by breast cancer cells appear to be another means besides release of the soluble L1 ectodomain that breast cancer cells can use to autocrine stimulate their motility. Our PMA experiments reveal that release of the L1 ectodomain can be modulated within the cells. Besides using our established *in vitro* motility assays to quantitated breast cancer cell behavior, our *in vivo* chick embryo model is sensitive and can be used to detect changes in metastatic ability that result from modulation of L1.

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Appendices

2008 Era of Hope Meeting Abstract- follows.

2008 Era of Hope Meeting Poster- follows.

View Paper

NOVEL QUANTITATION OF AUTOCRINE STIMULATION OF CELL MOTILITY IN VITRO AND METASTASIS IN VIVO OF HUMAN BREAST CANCER CELL LINES

BC063323

Deni S. Galileo, Kalyani Chilukuri, Yupei Li, and Katy Teixeira

University of Delaware

This work explores the undescribed effects of different forms of the L1 adhesion/recognition molecule on breast cancer cell motility. Breast cancer cells metastasize to a variety of secondary sites, including brain, which is ultimately responsible for patient death. A recently discovered mechanism of proteolysis or “shedding” of the L1 adhesion molecule extracellular domain (ectodomain) results in autocrine/paracrine stimulation of motility in ovarian and uterine cancers and causes of high mortality rates. L1 is expressed and shed abnormally in some breast cancer cells along with cognate integrin receptors, so such an autocrine mechanism is now being explored. Also, breast cancer cells entering into the brain environment rich in axonal L1 may stimulate spreading in the brain, and may contribute to the 50% mortality of patients with brain metastases. This is an unexplored concept in the progression of breast cancer, and these studies are highly likely to provide new insights into the aspects of this disease that contribute to patient mortality. This novel mechanism may account for the ability of human breast cancer cells to express and shed the L1 adhesion molecule as a means to stimulate their own motility and metastasis. L1 expression in several commercially available breast cancer cell lines is being documented by western blot analyses using several antibodies to different L1 regions (i.e., ectodomain versus cytoplasmic domain). L1 expression in individual cells is being documented by immunocytochemistry and flow cytometry. Cell lines have been found that either do or do not express L1. In vitro motility analysis of cell behavior will be done using a novel automated time-lapse microscopy system and quantitation software. Data will be compared for cells in which (1) L1 expression has been attenuated by retroviral antisense, (2) the shed L1 ectodomain is sequestered by L1-blocking antibodies, (3) integrin receptors are blocked by RGD-containing L1 peptides, and (4) L1 ectodomain is overexpressed by retroviral vectors. In addition, fluorescently labeled cells will be tracked on cell monolayers that stably express cell surface (non-shed) or shed L1 to determine the effects of different forms of exogenous L1 (like on axon tracts). In vivo studies will involve a novel chick embryo brain tumor model but where drug-resistant cells are microinjected into the blood vessels of the chorioallantoic membrane (CAM). Several days later, brains will be dissociated, plated, and drug selected for resistant cancer cell colonies. This will precisely quantitate the number of breast cancer cells that localized to brain. This assay could then be used to determine metastatic potential between different cell lines, as well as with cell lines that have been experimentally treated (e.g., antisense-L1 infected). These approaches will determine if different known forms of L1 stimulate or attenuate breast cancer motility and metastasis. Correlation of results between these two complementary approaches will establish if the novel motility assay can predict metastatic or invasive potential. Therapeutic strategies can be devised to inhibit signals resulting from this mechanism that may contribute significantly to breast cancer cell spread and metastasis to brain and elsewhere.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0625.

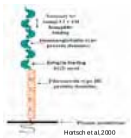
Role of L1-CAM in Stimulating Human Breast Cancer Cell Motility *In Vitro* and Metastasis *In Vivo*

Deni S. Galileo, Kalyani Chilukuri, Yupei Li, Kathryn Teixeira; **University of Delaware**

Abstract

The present work explores different forms of L1-CAM's potential effects in stimulating cancer cell motility and metastasis. Breast cancer cells can metastasize to a variety of secondary sites, including brain, which usually leads to ultimate patients' death. A recently discovered mechanism of proteolysis or "shedding" of cell adhesion molecule L1 reveals a pattern of autocrine/ paracrine stimulation of cell motility in ovarian and uterine cancers, resulting in high mortality rates. L1 is expressed and shed abnormally in some breast cancer cells, stimulating their motility and metastasis via interaction with focal adhesion molecules, such as integrins and FGF receptors. L1 is also released into the culture media in the form of exosomes. The biological significance of the secretion of exosomes from cancer cells is not known and they might add to the metastatic potential of cancer cells. L1 is found to be overexpressed in highly metastatic cell lines. Lentiviral vectors are used for overexpressing different fragments of L1 ectodomain in the less metastatic cell lines and shRNA lentiviral vector for downregulating L1 expression in highly metastatic cell lines. *In vitro* motility analysis of cell behavior will be done to examine the differences resulted thereafter. *In vivo* studies involve a novel model of chick embryo brain tumor forming assay, which will precisely quantitate the number of breast cancer cells that localize to brain and then to determine metastatic potential between different cell lines. Correlation of results between these two complementary approaches will establish if the novel motility assay can predict metastatic or invasive potential. Therapeutic strategies can be devised to inhibit signals transducted from this mechanism which may contribute significantly to breast cancer cell spread and metastasis, including to brain and elsewhere.

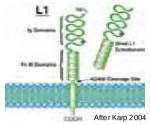
Background / Introduction



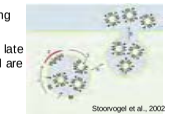
L1 Cell Adhesion Molecule

- L1 is the founder member of a subfamily of cell adhesion molecules that are primarily expressed in the nervous system.
- L1 can mediate cell–cell adhesion through Ca^{2+}-independent homo- or heterophilic binding at the cell surface.
- L1 is a 200-220 kDa glycoprotein.
- L1 contains six Ig-like domains linked to five fibronectin type III (FNIII) domains on the extracellular surface, a single-pass transmembrane domain and a short cytoplasmic tail of 110 residues.
- The major integrin-binding site of L1 lies in the sixth Ig domain which includes the consensus integrin binding motif RGD.
- The Ig2 domain is important for homophilic L1-L1 interactions.
- Neuronal and non-neuronal isoforms of L1 are formed by alternative splicing.
- Non-neuronal isoform of L1 is abnormally expressed in several cancers including breast, lung, ovarian, uterine, renal, endometrial, colon carcinomas.

Soluble L1



- Soluble L1 retains the ability to serve as substrate for integrin-mediated cell adhesion and migration
- Two forms of soluble L1 have been found in the cancer cells:
1. Shed ectodomain
2. L1 released in the form of exosomes
- L1 is cleaved by proteases like ADAM10 or ADAM17 and the shed ectodomain is released.
- Many cancer cells secrete exosomes, found to play a role in facilitating motility and metastasis.

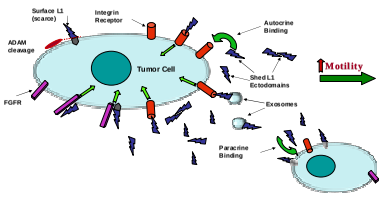


- Exosomes are 40-100nm membranous vesicular particles that form within late endocytic compartments, which are called multivesicular bodies (MVB), and are secreted upon fusion of these compartments with the plasma membrane.

Breast Cancer Metastases

- Breast cancer metastasizes to multiple sites including lung, liver, bone and brain, which can all be fatal.

Model of L1 stimulating breast cancer cell motility



L1 on breast cancer cell membrane will interact with other cells or factors in the ECM by autocrine and/or paracrine fashions to increase cell motility shown as above:

- L1 gets cleaved on cell membranes or in exosomes by ADAM protease family.
- L1 is released in the form of exosomes or shed ectodomain into culture media and affects cell motility.
- Shed L1 interacts with membrane integrins/ FGF receptor in autocrine/ papracrine fashoin.
- Shed L1 ectodomain forms homo- or heteropolymers to enhance cell motility.
- Uncleaved L1 interacts with integrins/ FGF receptors on cell membrane.

Results

In vitro Study on Role of L1 ---- Expression and Virus Construction

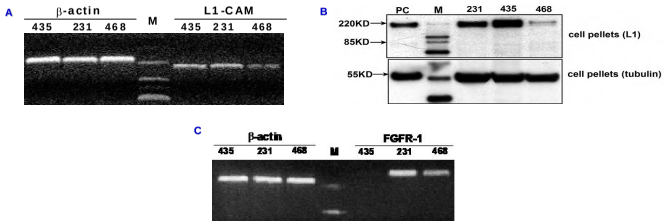


Figure 1. L1 expression level is tightly correlated with the metastatic ability of breast cancer cell lines.

A) RT-PCR result showing L1-CAM's expression in 231- MDA-MB-231; 435- MDA-MB-435 and 468- MDA-MB-468 cell lines, with beta-actin as the internal control.

B) Western blot analysis of full length L1 (220KD) and plasmin cleaved L1 cytoplasmic tail (85KD) expression in cell pellets of the same three breast cancer cell lines, with tubulin as the internal control. The least metastatic cell line MDA-MB-468 showed the least L1 expression. PC- U87 Glioma cells.

C) FGFR expression in breast caner cell lines tested. Besides MDA-MB-435 different from the other two as duct carcinoma, FGFR expression in MDA-MB-231 and 468 is correlated with L1-CAM.

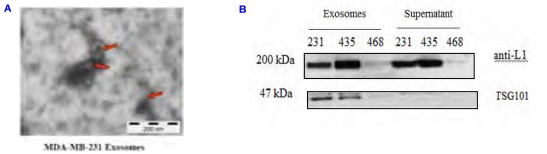


Figure 2. L1-CAM expression in exosomes.

A) Transmission electron microscopy images of exosomes isolated from the media of MDA-MB-231 cells as an example. The isolated exosomes can be visualized as small 40-100 nm membrane vesicles. (Unpublished data from Shalini Adla)

B) Western blot showing L1 expression in the exosomal pellets and cell supernatant of MDA-MB-231, MDA-MB-435 and MDA-MB-468 cells. Exosomes are isolated from the cells by differential centrifugation. Presence of exosomes is confirmed by checking for the exosomal marker TSG101. Exosomal L1 is expressed in low level in MDA-MB-468 (less metastatic) cell line.

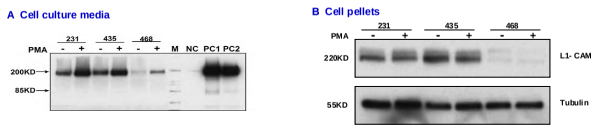


Figure 3. PMA induces L1 shedding by ADAM10 into the media.

A) L1 shedding analysis . MDB-MA-231, MDA-MB-435 and MDA-MB-468 cells were stimulated by 100ng/ml PMA in serum free media for 1h, cell culture medium was then precipitated by TCA overnight to get protein pellets for western blot analysis. Shed L1 ectodomain (200KD) were shown in the three cell lines. The least metastatic cell line MDA-MB-468 showed the least L1 ectodomain shedding. Cell pellets from NC- QT6 cells; PC- U87 Glioma cells.

B) Induced L1 shedding by PMA did not affect overall L1 expression. L1 expression was checked in cell pellets in the three cell lines, no obvious increase was shown in any of the cell lines. Tubulin was used as internal control.

Figure 4. Using shRNA to knock down L1-expression in highly metastatic cells.

A) Linear schematic diagram of lentiviral vector pLKO.1 from Open Biosystems.

B) Western blot showing the L1 expression in MDA-MB-435 cells and MDA-MB-435 infected with L1- shRNA-expressing lentiviral vector, with tubulin as internal control. As shown, L1 expression is greatly depressed in infected MDA-MB-435 cells.

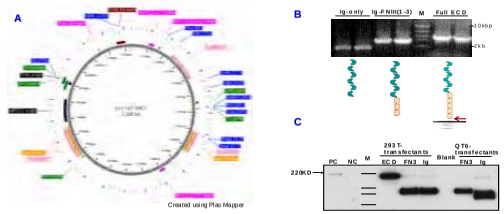


Figure 5. Lentivirus vector construction of different L1 ectodomain fragments and their expression.

A) Schematic diagram of the structure of Lentivirus LVV-1879MCX being used. Different L1 ectodomain fragments were cloned respectively into MCS region by SpeI and Xho I restricted enzyme sites.

B) PCR cloning of L1 ectodomain fragments. Full ECD is the whole L1 ectodomain without cytoplasmic tail, mimicking ADAM10 cleavage; Ig-FNIII stands for full Ig domain and 1-3 type III FN domain, and Ig-only is L1 ectodomain without any FNIII repeats.

C) Infection and expression of constructed L1-ectodomain lentiviruses in 293T and QT6 cells. Shown as above, the three fragments were expressed successfully in 293T or QT6 cell lines. NC- plain QT6 cells w/o L1 infection; PC- U87 Glioma cells.

In vivo Study on L1 stimulating Breast cancer metastasis to brain

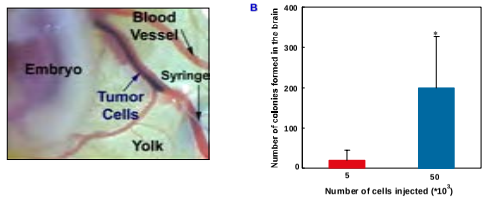


Figure 6. Quantative analysis of L1 stimulating breast cancer cell metastasis to brain in chick embryos model.

A) Microinjection of MDA-MB-231 into chick embryos. Cells were mixed with Fast Greeen and injected into thevasculature of E5 chick embryo via nanomanipulator.

B) Histogram showing the clones successfully formed in chick brains after breast cancer cell injection. 5,000 or 50,000 MDA-MB-231 cells were used. Several days later, brains were dissociated, plated, and the colonies formed in dissected chicks were shown. P< 0.05

Conclusions and Future Work

Conclusions so far

- *In vitro*, L1 expression is highly correlated with breast cancer cell lines' metastatic ability, with highly metastatic one expressing high level of L1 and vice versa; Also, FGFR expression is correlated with L1 expression levels suggesting potential interaction.
- L1 can be cleaved by ADAM10 either in the exosomes or on cell membrane to release the ectodomain into cell culture media. In both cases, the expression pattern is positively correlated with cell lines metastatic ability; PMA could induce L1 shedding by ADAM10.
- Lentiviral vectors expressing different L1 ectodomain fragments were successfully constructed and expressed in tube cells; L1 expression can be effectively knocked down by specific shRNA expressing lentivirus.
- *In vivo*, L1 can facilitate breast cancer cell metastasis into brain. At limited cell number of 5,000, new tumor colonies can still be formed.

Future Work

- To analyze and correlate levels of L1 expression with cell motility by performing cell function assays such as migration, cell invasion etc. using time-lapse microscopy.
- To introduce L1 into less metastasis cell lines and compare cell motility difference before and after; both in vitro and in vivo.
- To examine L1's interaction with integrin or FGF receptors on cell membrane and possible down stream signal transduction pathways.

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